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Ribosome-induced Dissociation of RNA from an RNA Polymerase–DNA–RNA Complex

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RNA, synthesized with RNA polymerase and T7 DNA as template, remains bound in a complex with enzyme and the DNA. The complex of RNA polymerase–T7 DNA–RNA, in contrast to one formed from enzyme and T7 DNA alone, is not dissociated in 0·5 M-NaCl but it is destroyed after denaturation of the enzyme with sodium dodecyl sulfate. If purified 70 S ribosomes are added to the enzyme–DNA–RNA complex, approximately 50 to 70% of the RNA in the complex is removed and sediments with or slightly faster than the 70 S ribosomes; little or none of the T7 DNA becomes associated with the fast-sedimenting ribosome–RNA entity.

1. Introduction

Wood & Berg (1962) and later others (Ning & Stevens, 1962; Furth, Kahan & Hurwitz, 1962; Doerfler, Zillig, Fuchs & Albers, 1962; Byrne, Levin, Bladen & Nirenberg, 1964; Bladen, Byrne, Levin & Nirenberg, 1965; Eiserling *et al.*, 1964) showed that supplementing the usual soluble protein–ribosome system with DNA, RNA polymerase and the appropriate nucleoside triphosphates resulted in a marked increase in protein synthesis. Several lines of evidence indicated that it was the RNA synthesized by the RNA polymerase which was responsible for the stimulation of protein synthesis. Particularly striking was the finding that RNA, synthesized in the presence of the soluble protein–ribosome components, was more effective in stimulating amino acid incorporation than was isolated RNA (Wood & Berg, 1962).

It was of interest, therefore, to learn how enzymically synthesized RNA becomes associated with ribosomes to permit protein synthesis. Bremer & Konrad's finding (1964), that RNA synthesized with RNA polymerase becomes part of a complex containing the enzyme and the DNA template, suggested that complexed RNA might associate with ribosomes more efficiently than free RNA. Conceivably, ribosomes or protein synthesis itself are needed to remove the newly formed RNA from the enzyme–template complex (Stent, 1965). Accordingly, purified 70 S ribosomes were added to the ternary complex (i.e. RNA–DNA–RNA polymerase) formed with T7 DNA as template and the fate of the newly made RNA followed by sucrose gradient

centrifugation. Between 50 and 70% of the RNA formerly in the complex sedimented with or slightly ahead of the 70 s ribosomes. Little or none of the T7 DNA was associated with the ribosome–RNA peak.

2. Materials and Methods

Escherichia coli B was used for the preparation of ribosomes and soluble protein fractions, for the isolation of RNA polymerase (Chamberlin & Berg, 1962) and for preparing labeled and unlabeled T7 phage. Unlabeled ribonucleoside triphosphates were obtained from Pabst Laboratories, Milwaukee, Wisc. [^{32}P]GTP (85% pure judged by electrophoresis) was purchased from International Chemical and Nuclear Corporation, City of Industry, Calif.; [2-^{14}C]ATP was bought from Schwartz BioResearch, Inc., Orangeburg, N.Y. and [methyl-^{3}H] thymidine from New England Nuclear Corporation, Boston, Mass.

Sucrose for gradient sedimentation was purchased from Mann Research Laboratories, Inc., New York, N.Y., filtered through Millipore filters and stored under aseptic conditions. Phenol (Mallinckrodt Analytical Reagent), 88% was distilled and stored frozen in the dark. Sodium dodecyl sulfate was recrystallized from 95% ethanol. Cesium sulfate and cesium chloride were obtained from Harshaw Chemical Company, Cleveland, Ohio.

Glass filters, type GF/C and Whatman 3MM circular discs, 2·5 cm in diameter, were obtained from H. Reeve Angel and Company, Inc., New York, N.Y.

M13 bacteriophage DNA (Salivor, Tzagloff & Pratt, 1964) was a generous gift from Dr S. Slapikoff.

(a) *Preparation of T7 phage and isolation of T7 DNA*

10 ml. of an overnight culture of *E. coli* B in Tryptone broth were transferred to 300 ml. of M9 medium containing 0·8% glucose (Adams, 1959). The culture was incubated at 37°C on a gyratory shaker and growth was followed by turbidity measurements at 595 mμ. When the absorbance reached 1·2 to 1·4, T7 (7×10^{10} phage/ml.) was added to the culture at a multiplicity of 0·1. Incubation was continued at 37°C and lysis, which usually was complete in 100 to 120 min, was followed by measuring the decrease in turbidity. After centrifugation at 4°C for 10 min at 9000 rev./min, the supernatant fluid was decanted into a sterile flask. Subsequent operations were performed aseptically. The lysate, containing $3{\cdot}4 \times 10^{11}$ phage particles/ml., was centrifuged for 45 min at 30,000 rev./min and 4°C in a no. 30 rotor of the Spinco model L preparative ultracentrifuge. Pellets were suspended in 10 ml. of a 0·01 M-Tris buffer, pH 7·9 to 8·0, containing 1 M-NaCl (Tris–NaCl), centrifuged again for 10 min at 9000 rev./min to remove debris. The pellet was resuspended in 1·0 ml. Tris–NaCl and re-centrifuged. The first supernatant fluid and wash were combined, centrifuged for 15 min at 40,000 rev./min in the Spinco ultracentrifuge, and the pellet was suspended in 1·0 ml. of Tris–NaCl. Further purification of the phage was achieved by centrifugation in a three-layered gradient of buffered CsCl added as follows. The first layer was 1 ml. of the phage suspension, the next layer was 1·5 of 0·33 saturated CsCl, the next layer was 1·5 ml. of 0·50 saturated CsCl and the top layer was 1·5 ml. of 0·67 saturated CsCl. The tubes were centrifuged in the SW39 swinging bucket rotor in the Spinco model L for 2 hr at 35,000 rev./min and 4°C. The phage band which was 1·8 to 2·0 cm from the bottom of the tube was collected by puncturing a hole in the bottom and collecting the drops. DNA was extracted from the phage (15 to 20 A_{260}/ml. in 0·01 M-Tris, pH 8·0) by very gently shaking with phenol (saturated with 0·1 M-Tris, pH 8·0): 3 to 4 extractions were usually sufficient to eliminate protein from the interphase. The DNA solution was dialyzed against 0·01 M-Tris, pH 8·0, to remove phenol. The final preparation, which had an $A_{260/280}$ of 1·85 to 1·93 was then stored at 0°C. The same procedure was used to prepare ^{3}H-labeled T7 DNA. In such cases, 1·5 mc of [^{3}H]thymidine were added to the growing culture 10 min after infecting with phage. Otherwise, all steps involved were as described above.

T7 DNA, isolated as described above, sediments as a single boundary at neutral pH as judged by centrifugation in a preparative sucrose gradient (Fig. 1) and zone sedimentation (Vinograd, Bruner, Kent & Weigle, 1963) in the analytical centrifuge (Fig. 2(a))

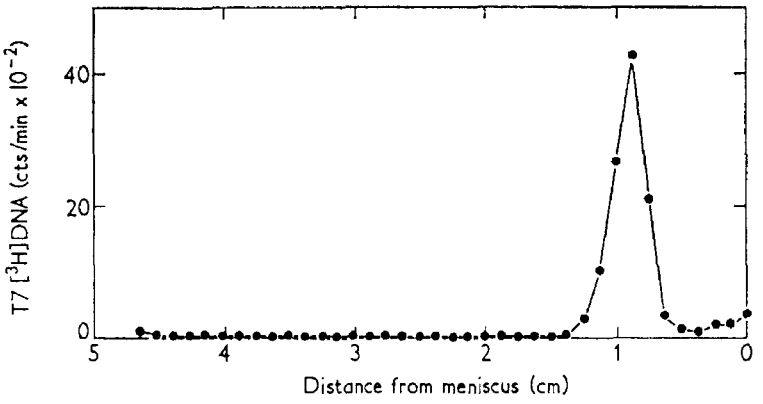

FIG. 1. Sedimentation of T7 [^{3}H]DNA in sucrose gradient (5 to 20%) containing 0·05 M-Tris buffer, pH 8·0. 2 μg T7 [^{3}H]DNA (approximately 10,000 cts/min) were incubated at 37°C for 10 min in a solution (0·25 ml.) containing 10 μmoles Tris buffer, pH 8·0, 1 μmole $MgCl_2$ and 3 μmoles 2-mercaptoethanol. The entire reaction mixture was layered on a preformed gradient and centrifuged for 3 hr at 25,000 rev./min and 3·5°C (rotor temperature) in the SW39 rotor. Drops were collected and assayed as described in Materials and Methods. Recovery of ^{3}H counts was 98%.

(see also earlier studies of Studier, 1965 and Davison & Freifelder, 1962). Inclusion of $MgCl_2$ (up to 0·01 M) in the gradient has no effect on the shape or position of the band or on the recovery of the T7 [^{3}H]DNA (98%). At alkaline pH (Fig. 2(b)) there is very little trailing material, indicating that at least 85 to 90% of the single strands are intact and presumably less than one break per two native T7 DNA helices.

After incubation of T7 DNA with and without purified RNA polymerase (weight ratio of DNA to enzyme = 2), alkaline zone centrifugation of the recovered DNA gives essentially the same pattern (shown in Fig. 2(b)). In another test for DNase in the enzyme preparation, RNA polymerase (200 μg) was incubated with the single-strand rings of phage

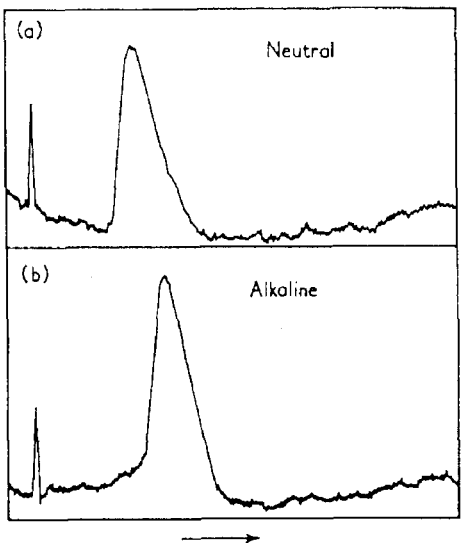

FIG. 2. Zone sedimentation of T7 DNA in 1 M-salt at neutral and alkaline pH. The zone sedimentation technique was performed as described by Studier (1965), with a centrifugation speed of 25,980 rev./min. Pictures were obtained at 4-min intervals and tracings of the sedimentation pattern were made with a Joyce–Loebl microdensitometer from the 12-min photographs. Sedimentation was carried out at pH 8·0 (a) and in 0·1 M-NaOH (b).

M13 DNA (Salivor *et al.*, 1964), for 20 min at 37°C and after deproteinization with phenol, and overnight dialysis, the DNA was examined by zone sedimentation in 0·01 M-NaOH–0·9 M-NaCl in a model E ultracentrifuge (Vinograd *et al.*, 1963). No degradation of the ring structure was observed as evidenced by the equal amounts of open rings in the incubated sample and the control without RNA polymerase. These results indicate that under the conditions of our experiments (ratio of enzyme to DNA and absolute quantity of RNA polymerase used) there is no detectable DNA endonuclease activity.

(b) *Preparation of RNA polymerase*

RNA polymerase preparations were either fraction 4 described by Chamberlin & Berg (1962) or a fraction which was purified further by sucrose gradient centrifugation as follows: the peak fractions obtained by DEAE chromatography (Chamberlin & Berg, 1962) were pooled and precipitated with saturated ammonium sulfate, and dissolved in approximately 2 ml. of "freezing buffer" (Chamberlin & Berg, 1964). The enzyme solution was adjusted to 0·01 M-2-mercaptoethanol and layered on a preformed sucrose gradient (10 to 20%) containing 0·001 M-EDTA, 0·01 M-2-mercaptoethanol and 0·5 M-NaCl in 0·01 M-Tris buffer, pH 8·0. Centrifugation was performed for 30 hr at 25,000 rev./min and 2°C in an SW25·1 swinging bucket rotor. Drops were collected through the bottom and assayed for RNA polymerase activity (Chamberlin & Berg, 1962). The enzyme contained within the peak was precipitated with ammonium sulfate (0·5 saturated), dissolved in "freezing buffer" and stored in liquid nitrogen. Specific activities of typical preparations ranged from 3000 to 4100.

(c) *Preparation of ribosomes*

40 g of *E. coli* B, harvested in logarithmic growth phase, were suspended in 100 ml. Tris buffer 0·01 M, pH 7·8, containing 0·008 M-magnesium acetate and 0·06 M-KCl. Cells were disrupted in a French pressure cell (20,000 lb. pressure on a 2·563-in. ram). Thereafter, all procedures were performed at 3 to 4°C. The crude extract was centrifuged for 30 min at 12,000 ***g***. The supernatant solution was decanted and centrifuged for another 30 min at 30,000 ***g***. To the supernatant fluid was added magnesium acetate to a final concentration of 0·015 M and glutathione (neutralized) to a final concentration of 0·002 M.

14·6 g solid ammonium sulfate were added to 85 ml. of the supernatant fraction with stirring. After 5 min the suspension was centrifuged for 10 min at 12,000 ***g*** and the supernatant fluid was dialyzed overnight against 3 changes of 3 l. 0·01 M-Tris buffer, pH 7·8, containing 0·008 M-magnesium acetate and 0·06 M-KCl.

The dialyzed supernatant was centrifuged for 2 hr at 105,000 ***g*** in a Spinco ultracentrifuge and the ribosome pellets were suspended in a solution (1/10th original volume) containing 0·01 M-Tris, pH 7·8, 0·01 M-magnesium acetate, 0·06 M-KCl and 0·006 M-2-mercaptoethanol and then centrifuged at 30,000 ***g*** to remove debris.

Ribosomes were further purified as follows: 1 ml. ribosomes were layered on 7 ml. 10% sucrose solution containing 0·01 M-Tris, pH 7·8, 0·01 M-magnesium acetate and 0·006 M-2-mercaptoethanol and 0·5 M-NH_4Cl (Voorma & Bosch, 1965) and centrifuged for 90 min at 60,000 rev./min in an International 695 preparative ultracentrifuge. The clear ribosome pellet was suspended in 1 ml. of Tris buffer 0·01 M pH 7·8 containing 0·01 M-magnesium acetate, and 0·001 M-glutathione centrifuged for 5 min at 12,000 ***g*** and dialyzed overnight (2 changes) against 2 l. of the same buffer. Ribosomes were stored in liquid nitrogen at a concentration of 12 to 15 mg protein/ml.

50 s and 30 s subunits were prepared according to the method described by Takanami & Okamoto (1963). Following dialysis against $2·5 \times 10^{-4}$ M-magnesium acetate the ribosomes were fractionated by repeated centrifugation in a sucrose gradient (6 hr at 25,000 rev./min and 4°C in an SW25·1 rotor). Separate fractions containing the 50 s and 30 s subunits were pooled and centrifuged again for 8 to 12 hr at 40,000 rev./min. The clear pellets were suspended in 0·01 M-Tris, pH 7·8, containing 0·001 M-glutathione and $2·5 \times 10^{-4}$ M-magnesium acetate and stored in a liquid-nitrogen refrigerator.

70 s ribosomes were reconstituted from 50 and 30 s subunits by dialysis against 5 changes of 0·01 M-Tris, pH 7·9, 0·015 M-magnesium acetate and 0·006 M-2-mercaptoethanol at 4°C for 30 hr.

(d) *Assays*

RNA polymerase was assayed as described by Chamberlin & Berg (1962). Reactions were terminated by the addition of 0·2 mg bovine plasma albumin and 2·0 ml. of cold perchloric acid. The samples were filtered through Whatman GF/C glass discs (2·4 cm diameter); the discs were washed with about 30 ml. of cold 2 N-HCl, then with about 15 ml. 95% ethanol, dried and counted in a Nuclear Chicago liquid-scintillation spectrometer.

Reaction mixtures (0·25 ml.) analyzed by centrifugation through sucrose gradients contained, unless otherwise specified, 10 μmoles of Tris buffer, pH 8·0, 1·0 μmole of $MgCl_2$, 50 mμmoles each of ATP, UTP, GTP, and CTP, one of which was labeled with either ^{32}P or ^{14}C, and 3·0 μmoles of 2-mercaptoethanol. The amount of RNA polymerase and DNA added to the reaction is indicated in each experiment. At various times of incubation, a portion to measure the amount of RNA synthesis was removed and the remaining reaction mixture was layered on a preformed sucrose gradient.

Sucrose gradient centrifugation (5 to 20% sucrose w/v) was performed in either an SW39 (4·9 ml. final vol.) or SW25·1 (30 ml. final vol.) swinging bucket rotor maintained at 6 and 3°C, respectively, during centrifugation. Drops were collected from the bottom of each tube, and usually alternate fractions were assayed for T7 [^{3}H]DNA, ^{32}P- or ^{14}C-labeled (newly synthesized RNA) and A_{260} (ribosomes) in either of two ways.

(1) 2-drop fractions (less than 0·15 ml.) were collected directly on Whatman 3MM discs (2·5 cm in diameter) which were then immediately immersed in a beaker containing cold 5% trichloroacetic acid (50 discs/l.). The acid solution was stirred periodically for 15 min, the trichloroacetic acid decanted and fresh cold trichloroacetic acid added. After 2 washes, the discs were rinsed first in a solution of ether–ethanol (1 : 1) for 3 to 4 min, followed by ether for a few minutes. The ether was decanted, the discs dried and put into scintillation vials for counting. (2) Fractions from sucrose gradients were collected in tubes and the nucleic acid precipitated with 2·0 ml. 7% perchloric acid recovered by filtration through GF/C glass filters, washed with HCl, then ethanol, dried and counted.

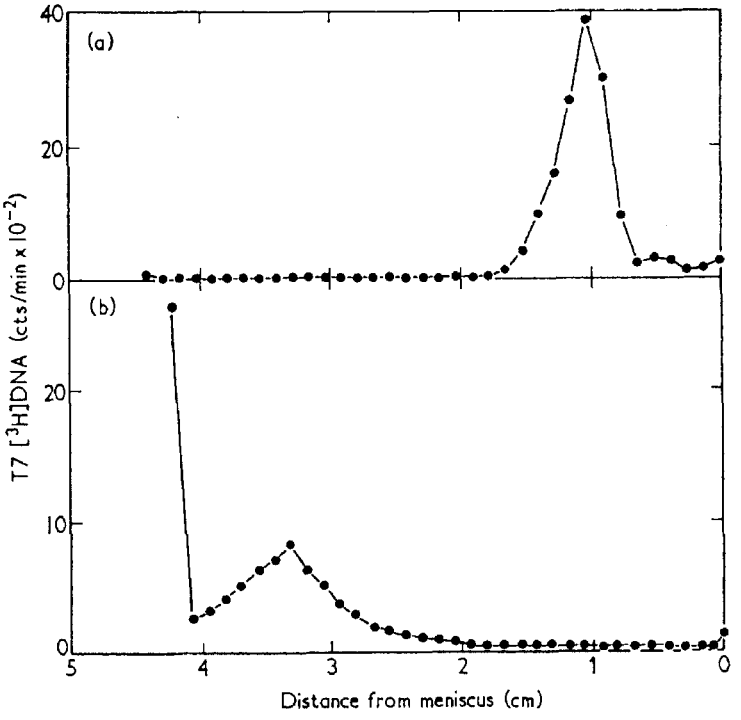

FIG. 3. Effect of RNA polymerase on sedimentation behavior of T7 DNA. All centrifugations were in the SW39 rotor at 25,000 rev/min for 3 hr and 3·5°C. (a) 2 μg T7 [^{3}H]DNA (approximately 20,000 cts/min) was incubated with 1 μg RNA polymerase in the buffer described in Fig. 1 for 10 min at 37°C and then analyzed as in Fig. 1. In (b) the conditions were the same, except that the 15 μg of enzyme were added.

(e) *General methods*

All counting procedures were performed in a Nuclear Chicago liquid-scintillation spectrometer. For dual-label counting using ^{3}H and ^{32}P, less than 0·5% of the tritium counts appeared in the ^{32}P channel so this overlap was ignored. 2 to 3% of the ^{32}P counts appeared in the ^{3}H channel and appropriate corrections were made.

All optical density measurements were made in a Zeiss PMQ11 spectrophotometer.

3. Results

(a) *Formation of a complex with T7 DNA and RNA polymerase*

When DNA and increasing quantities of RNA polymerase are mixed and centrifuged in a sucrose gradient, there is a progressive increase in the sedimentation velocity and polydispersity of the DNA. When the weight ratio of DNA to enzyme is 2·0, the displacement is slight (Fig. 3(a)) compared to that found for DNA alone (Fig. 1), but when the ratio is 0·14, the sedimentation pattern is strikingly altered (Fig. 3(b)) and when the ratio is 0·07 all of the DNA sediments to the bottom of the tube (not shown).

If sodium dodecyl sulfate (0·5%) or KCl (0·5 M) is added to the mixture of enzyme and DNA, the sedimentation behavior of the DNA is indistinguishable from that of free

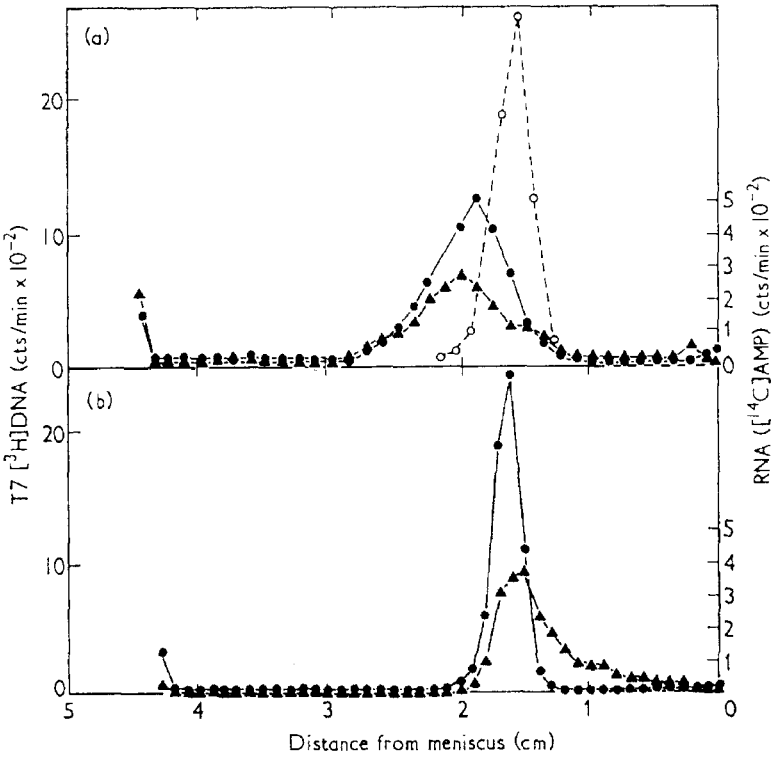

FIG. 4. Sedimentation of a RNA polymerase–DNA–RNA complex and its disruption by sodium dodecyl sulfate. RNA synthesis was continued for 10 min as described in Materials and Methods, with 2 μg T7 [^{3}H]DNA, 1 μg RNA polymerase and with [^{14}C]ATP as the labeled triphosphate. Approximately 10,000 cts/min T7 [^{3}H]DNA and 2700 cts/min as acid-precipitable [^{14}C]AMP in newly synthesized RNA was layered on a preformed sucrose gradient containing 0·05 M-Tris–HCl, pH 8·0. In (a) the pattern for the synthesized complex is shown (solid curve) and superimposed on this is the profile of free DNA (dashed curve). In (b), is the profile of the synthesized complex after treatment with 0·5% sodium dodecyl sulfate for 3 min at 37°C. All runs were for 3 hr at 35,000 rev./min and 3·5°C. Drops were collected on Whatman 3mm paper discs and assayed as described in Materials and Methods. —●—●—, ^{3}H radioactivity; —▲—▲—, ^{14}C radioactivity.

DNA (Fig. 1). We assume that in sodium dodecyl sulfate or high salt concentration the complex is dissociated yielding free T7 DNA and protein (see Fig. 5, Jones & Berg, 1966).

(b) *Formation of a complex with RNA polymerase, T7 DNA and newly synthesized RNA*

After incubation of T7 DNA, RNA polymerase and four nucleoside triphosphates, the newly synthesized RNA sediments with the DNA (Fig. 4(a)). The relatively broad peak of DNA and RNA consistently sediments faster than does free DNA (dashed curve Fig. 4(a)). If sodium dodecyl sulfate is added to the synthesized complex the sedimentation behavior is altered and both the DNA and RNA sediment at the same rate as free DNA (Fig. 4(b)). This differs from the observation of Bremer & Konrad (1964), who observed that following treatment of an analogous synthesized complex (using T4 DNA) with sodium dodecyl sulfate, the RNA sedimented more slowly than the T4 DNA; this could be because the T7 DNA and the RNA remain complexed together even after denaturation of the RNA polymerase or because, under our conditions of synthesis, the complementary RNA and T7 DNA have about the same sedimentary coefficient.

The latter explanation seems more likely, since equilibrium sedimentation of the synthesized RNA in a $CsSO_4$ gradient shows that the bulk of the RNA is not complexed to the DNA; that is, it bands at the characteristic buoyant density of free RNA (Fig. 5). A small amount (10 to 15%) of synthesized RNA invariably appeared at a buoyant density of DNA 1·43 to 1·47. This fraction of the RNA may be complexed to DNA either in a hybrid structure with single-stranded DNA (Warner, Samuels, Abbott &

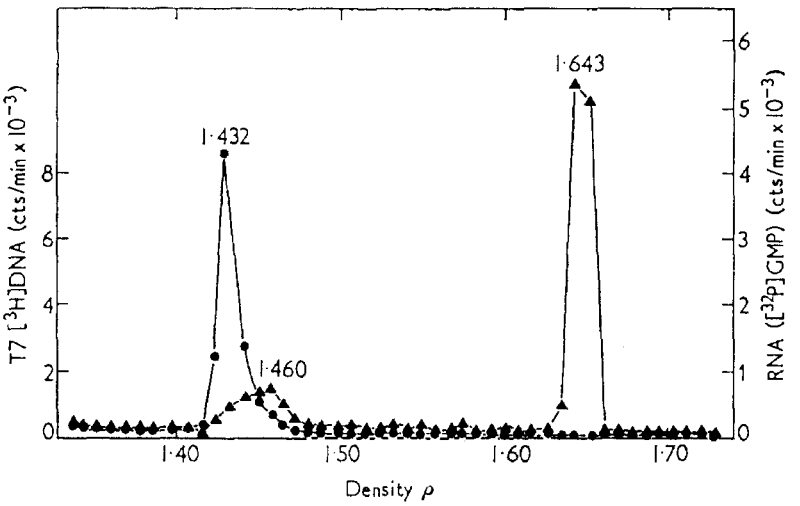

FIG. 5. $CsSO_4$ density-gradient analysis of synthesized complex containing T7 DNA–RNA after extraction with phenol. The reaction mixture (0·5 ml.) contained 4 μg T7 [^{3}H]DNA, 6 μg RNA polymerase, 150 mμmoles each of ATP, CTP and UTP and 150 mμmoles [^{32}P]GTP (specific activity of $1·8 \times 10^5$ cts/min/mμmole). Approximately 0·95 equivalent of RNA (relative to DNA) was synthesized. The reaction mixture was extracted with buffer-saturated phenol and dialyzed against 0·01 M-NaCl in 0·01 M-Tris, pH 8·0, and 0·001 M-EDTA for 18 hr. A portion containing approximately 16,000 cts/min T7 [^{3}H]DNA and 16,000 cts/min acid-precipitable [^{32}P]GMP in RNA, was made 42·8% in $CsSO_4$ (Chamberlin & Berg, 1964) and brought to a final volume of 3·0 ml. in 42·8% $CsSO_4$ containing 0·03 M-Tris-HCl, pH 8·0, and 0·001 M-EDTA. The sample was centrifuged at 33,000 rev./min and 8°C for 72 hr. Fractions were collected and assayed for acid-precipitable radioactivity and the density was determined by pycnometry. Recovery of [^{32}P]RNA and T7 [^{3}H]DNA was 75% and more than 95% respectively. —●—●—, ^{3}H radioactivity; —▲—▲—, ^{32}P radioactivity.

Krakow, 1963; Sinsheimer & Lawrence, 1964; Chamberlin & Berg, 1964) or possibly to double-stranded DNA as recently found by Hayashi (1965).

Further support for the conclusion that T7 DNA and the RNA product do not remain associated following treatment with sodium dodecyl sulfate is seen from the sedimentation behavior of the RNA isolated at various times during the synthesis. If RNA is removed after 0·5, 2 and 5 minutes of synthesis, i.e. during the linear phase of transcription, treated with sodium dodecyl sulfate, and then analyzed as in Fig. 4(b), the sedimentation rate of the free RNA is seen to increase as synthesis progresses (Fig. 6). In the absence of sodium dodecyl sulfate, the RNA and DNA sediment together as mentioned above, no matter how much synthesis has occurred. At early

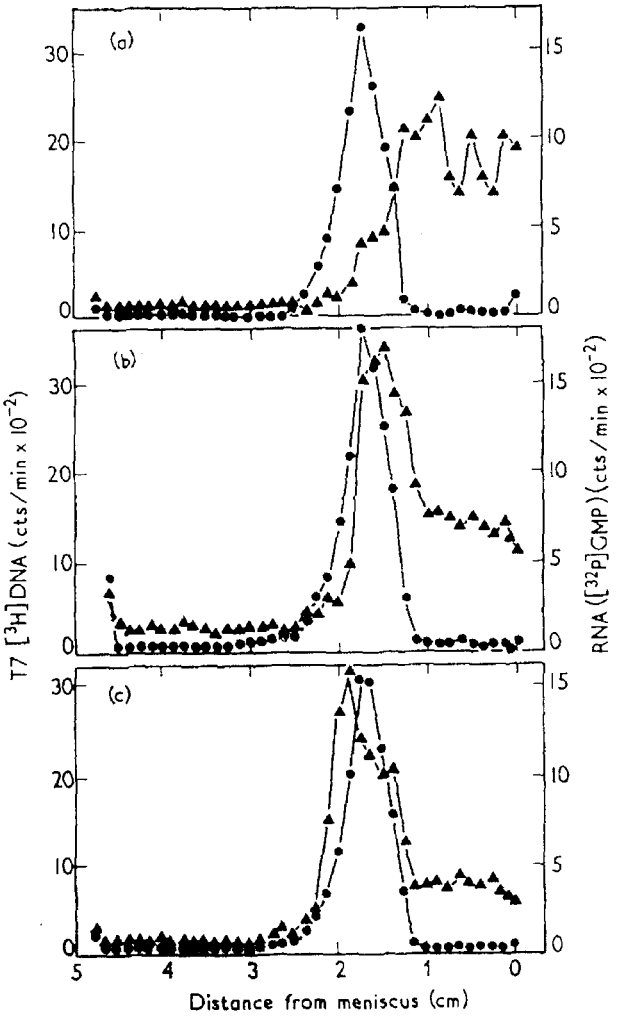

FIG. 6. Analysis of the sedimentation behavior of the RNA freed from the RNA polymerase DNA–RNA complex by sodium dodecyl sulfate, as a function of the extent of RNA synthesis. For these experiments, a T7 DNA to RNA polymerase weight ratio of 25 was used. Reaction mixtures for RNA synthesis were as described in Materials and Methods with [^{32}P]GTP ($3{\cdot}6 \times 10^4$ cts/min/mμmole). After 30 sec (a), 2 min (b), and 5 min (c) at 37°C portions were removed, made 0·5% in sodium dodecyl sulfate, incubated 3 min at 37°C and then layered on sucrose gradients containing 0·05 M-Tris buffer, pH 8·0. Samples, containing 0·33, 0·48 and 0·78 mμmole [^{32}P]GMP, respectively, in RNA, were centrifuged for 3 hr at 35,000 rev./min and 3·5°C and fractions were collected and assayed for acid-precipitable DNA and RNA as described in Materials and Methods. —●—●—, ^{3}H radioactivity; —▲—▲—, ^{32}P radioactivity.

stages of synthesis the sedimentation of the free RNA is considerably less than the free T7 DNA but as synthesis proceeds the sedimentation velocity of the RNA rapidly approaches and in several experiments exceeds that of the template.

(c) *Dissociation of DNA–enzyme–RNA complex by ribosomes*

Since earlier studies of protein synthesis suggested that RNA synthesized by RNA polymerase could direct amino acid incorporation (Wood & Berg, 1962), we examined the effect of added ribosomes on the enzyme–DNA–RNA complex.

If ribosomes (70 s) are added to the RNA polymerase–DNA–RNA complex, approximately 60% of the RNA, which sediments with T7 DNA, now sediments with or slightly ahead of the ribosomes peak (Fig. 7). Very little of the T7 DNA appears in the ribosome region (the profile of free T7 DNA and ribosomes is essentially the same as that seen in Fig. 7).

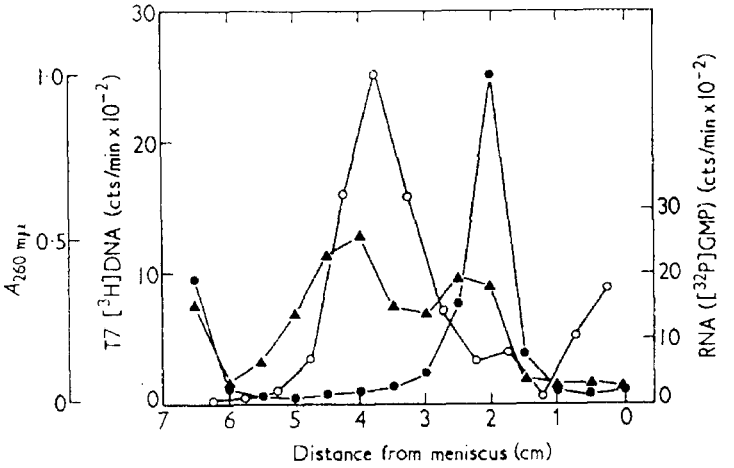

FIG. 7. The formation of a ribosome–RNA complex when 70 s ribosomes are added to the RNA polymerase–DNA–RNA complex. The RNA polymerase–DNA–RNA complex was prepared as described in Materials and Methods, using a T7 DNA to polymerase weight ratio of 25. After 20 min at 37°C, 2·0 mg of *E. coli* 70 s ribosomes were added to the reaction and after an additional 5 min at 37°C portions were removed for assay of acid-precipitable [^{32}P]RNA and [^{3}H]DNA. The remaining sample was layered on to a preformed sucrose gradient.

Centrifugation was performed in an SW 25·1 rotor at 25,000 rev./min for 6 hr at 3·0°C. Collection of fractions and precipitation on glass filters are described in Materials and Methods. —○—○—, Absorbance; —●—●—, ^{3}H radioactivity; —▲—▲—, ^{32}P radioactivity.

The quantity of RNA sedimenting with the ribosomes varies between 50 to 70% and does not increase with addition of more ribosomes or with longer times of incubation. We have observed, however, that the stage during transcription at which ribosomes are added does effect the proportion of the RNA which sediments with the ribosomes. When ribosomes are added early in transcription (after two minutes), approximately 10% of the RNA appears with the ribosomes (Fig. 8(a)), but when the RNA has reached maximum size (after 20 minutes) nearly half of the RNA becomes associated with ribosomes (Fig. 8(b)). This indicates that the RNA may have to reach a certain size before it can be removed from the enzyme–DNA–RNA complex by ribosomes.

It was of interest to determine if the entire 70 s ribosome was essential for dissociation of RNA from the synthesized complex. Figure 9(a) shows that little of the RNA in the RNA polymerase–DNA–RNA complex forms a complex with the 50 s ribo-

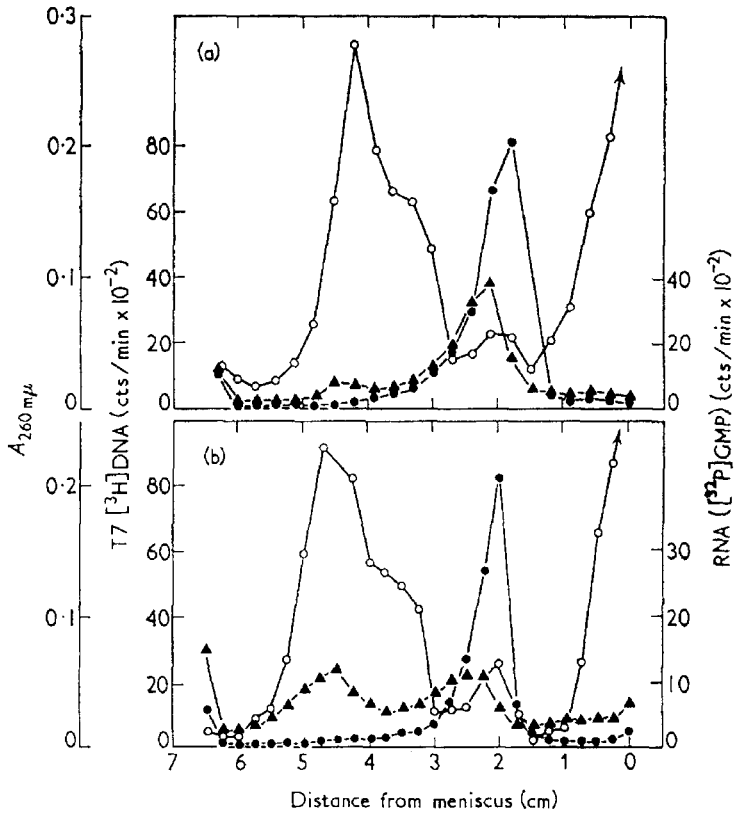

FIG. 8. Formation of ribosome–RNA complex from the RNA polymerase–DNA–RNA complex as a function of the extent of RNA synthesis. The RNA polymerase–DNA–RNA complex was prepared as described in Fig. 7. After 2 min (a) and 20 min (b) incubation, 2·5 mg *E. coli* ribosomes were added and incubation was continued for 5 min. The samples were then layered on preformed sucrose gradients and subjected to centrifugation for 6 hr at 25,000 rev./min in an SW25·1 rotor. Collection of fractions and of A_{260} acid-precipitable radioactivity is described in Materials and Methods. —○—○—, Absorbance; —●—●—, ^{3}H radioactivity; —▲—▲—, ^{32}P radioactivity.

somal subunit (or with ribosomal RNA) when it is substituted for the 70 S ribosomes. Since the enzyme–T7 DNA–RNA complex sediments at about 30 S, it was not possible to test the activity of 30 S ribosomes for their ability to dissociate the complex. Reconstituted 70 S ribosomes, made by mixing isolated 50 and 30 S ribosomal subunits, also removed RNA from the polymerase–DNA–RNA complex, although this activity was variable and the amount removed was smaller (<40%). This could be due to uncontrolled loss of a ribosomal component during the preparation of the 50 and 30 S subunits, which is needed for formation of a complex with the nascent RNA.

4. Discussion

The present studies with T7 DNA as template show, as did the earlier experiments with T4 DNA by Bremer & Konrad (1964) and Byrne *et al.* (1964), that RNA synthesized with RNA polymerase *in vitro* remains associated with the enzyme–DNA complex. The RNA–T7 DNA–RNA polymerase complex, in contrast to the complex of T7 DNA and RNA polymerase, is not dissociable by high (up to 1 M) salt concentration in the presence of excess polymerase. However, after denaturation of the enzyme with sodium dodecyl sulfate, free DNA and RNA are released.

A novel finding in this study was the ability of 70 s ribosomes to remove RNA from the RNA–DNA–enzyme complex. The addition of ribosomes to the complex led to the formation of a new complex of RNA and ribosomes which sedimented at or greater than 70 s and contained none of the DNA. The action of the ribosomes was therefore quite distinct from that observed with high salt concentration or sodium dodecyl sulfate.

Quite conceivably the explanation for this is that nucleases which might be in the ribosome preparations cleave RNA from the RNA–DNA–enzyme complex and this free RNA then reacts with ribosomes to form an RNA–ribosome complex. This does not seem likely since (1) the addition of ribosomes and continued incubation does not produce acid-soluble fragments from the RNA, (2) the transfer of RNA to the ribosomes occurs even if the mixture is maintained at 0°C before and during the sucrose gradient sedimentation, and (3) the labeled RNA recovered from the ribosome–RNA complex still sediments at about 25 to 30 s.

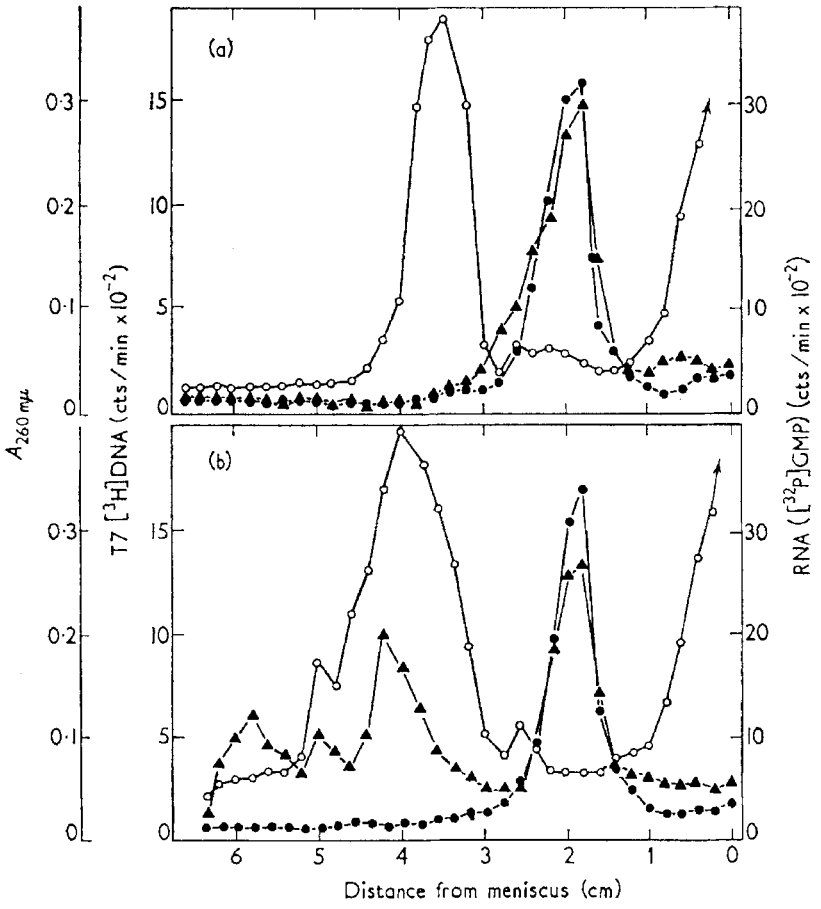

FIG. 9. Failure of the isolated 50 s ribosome subunit to remove RNA from the RNA polymerase–DNA–RNA complex. Reaction mixtures were prepared as described in Fig. 7. and after 20 min at 37°C, 50 s ribosomes subunits (a), in amounts equivalent to the 70 s fraction (see Fig. 7) and reconstituted 70 s ribosomes (b) were added. After 5 min more at 37°C the reaction mixtures containing 2·2 and 3·2 × 10^4 cts/min, respectively, as RNA were layered on a preformed sucrose gradient. Reconstituted 70 s ribosomes were prepared as described in Materials and Methods. Centrifugation was for 6 hr at 25,000 rev./min and 3°C in an SW25·1 rotor. Fractions were collected and assayed for A_{260} and acid-precipitable radioactivity as described in Materials and Methods. —○—○—, Absorbance; —●—●—, ^{3}H radioactivity; —▲—▲—, ^{32}P radioactivity.

Formation of a ribosome–RNA complex *in vitro* has been described by Byrne *et al.* (1964) (also see Bladen *et al.*, 1965). In their system, using T2 DNA as template, the ribosome–RNA complex also includes DNA. In our experiments, no significant amount of T7 DNA was found in the 70 s region. The difference in our results could be due to the different sources of template DNA (T2 *versus* T7) or to the fact that in their experiments the supernatant proteins and all the ingredients of protein synthesis were also present. On the other hand, we have shown that the weight ratio of RNA polymerase to template is critical and a relatively small excess of polymerase molecules is enough to result in a rapidly sedimenting aggregate with DNA (Fig. 3(b)). Byrne *et al.* (1964) showed that 84% of the fast-moving DNA moiety had an *S* value greater than 200 s; they suggested that the DNA molecules were associated with polysomes, but it is also possible that they observed complexes of DNA and RNA polymerase which sedimented at about the same rate as did the polyribosomes.

Since this work was completed, Shin & Moldave (1966) have also described a ribosome RNA complex following addition of 70 s ribosomes to a deoxynucleoprotein complex containing RNA polymerase and newly synthesized nascent RNA molecules. Their observations show that a portion of the new RNA is removed from the polymerase DNA–RNA complex, in keeping with our results.

Byrne *et al.* (1964) and Stent (1965) have suggested models for a coupled transcription–translation process whereby nascent messenger RNA molecules are removed from the template by ribosomes as they are synthesized. Although our finding of a ribosome-induced removal of RNA from the RNA–DNA–enzyme complex is superficially consistent with such a model, it should be pointed out that in our studies this occurred in the absence of protein synthesis. Because the supernatant enzymes were not added, protein synthesis *per se* could not have contributed to the removal of the RNA; this is also the most likely reason why the ribosome–RNA complexes found are monosomes and not polysomes (Haselkorn, Fried & Dahlberg, 1963; Shin & Moldave, 1966). The finding that 70 s ribosomes reconstituted from isolated 30 s and 50 s subunits show variable effectiveness in dissociating RNA from the RNA–DNA–polymerase complex may be rationalized by the recent reports (Revel & Gros, 1966; Brawerman & Eisenstadt, 1966; Eisenstadt & Brawerman, 1966; Stanley, Salas, Wahba & Ochoa, 1966) that at least one and possibly two "factors" are required to initiate translation of natural messenger RNA, and in the case of T2 RNA (Revel & Gros, 1966) the factor(s) are required in order for ribosomes to bind the RNA. It is possible that during isolation of 30 s and 50 s subunits, these factors are partially lost.

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